SPECTROSCOPIC EVIDENCE FOR INTERACTIONS OF MEROCYANINE 540 WITH VALINOMYCIN IN THE PRESENCE OF POTASSIUM

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Received 22 December 1979

1. Introduction

The application of optical probes to monitor membrane potential has attracted much attention in the past few years, particularly in biological systems where the cells are too small to be penetrated by conventional microelectrodes. Two common dyes which are often used are merocyanine 540 (MC-540) and the dye, 3,3′-dipropyl-2,2′-thiadicarbocyanine ($DiSC_3(5)$). These fluorescent probes have proved to be useful and sensitive for measuring membrane potential in many systems. Among them are squid giant axon [1], frog heart muscle [2] and erythrocytes [3–5]. The optical probes are frequently employed simultaneously with K^+ ionophore valinomycin [3,6,7]. In the human erythrocyte, valinomycin was used to impose certain membrane potentials in solutions of different $[K^+]$ [3,4]. A correlation between changes in fluorescence signal and membrane potential was established [3]. However, in view of the evidence for interactions between various organic compounds and K^+–valinomycin in cell-free medium [8–10], we feel it is important to test possible interactions between membrane potential fluorophores and ionophores in aqueous solution. We describe here by spectroscopic means the interaction between MC-540 and valinomycin in high K^+ medium. $DiSC_3(5)$ and valinomycin are shown not to interact. We report here how the difference between the two dyes is reflected in a biological system, like the human erythrocyte.

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2. Materials and methods

Venous blood was drawn from normal young males into a heparinized tube and was centrifuged at 1000 × *g* for 10 min at 4°C. The cells were washed 4 times with isotonic buffer containing 148 mM NaCl, 5 mM NaCl and 17 mM Tris–HCl at pH 7.4. Each wash consisted of centrifugation at 1000 × *g* for 10 min at 4°C. The dye were dissolved in ethanol. The final concentration of MC-540 (Eastman-Kodak Organic Chemical Co.) was 2.9 μM and of $DiSC_3(5)$ (generous gift from Professor Allan Waggoner of Amherst College) was 0.61 μM. The dyes were protected against light and kept in the cold. Valinomycin (Sigma Chemical Co.) was also dissolved in ethanol to final conc. 1 μM. The final concentration of ethanol in the cell suspension was generally 0.25% and never exceeded 0.5%. Fluorescence determinations were made with a Perkin-Elmer model MPF-4 spectrofluorimeter. The experiments were carried out at 26°C. Absorption studies were carried out with a Cary 11 spectrophotometer equipped with expanded scale. For circular dichroism (CD) measurements, a Cary model 60 spectropolarimeter with CD attachment (Cary 6001) was used.

3. Results and discussion

The effect of valinomycin on the absorption and emission spectra of MC-540 is demonstrated in fig.1(a,b). The medium contained 153 mM K^+. MC-540 has two equal absorption maxima, at 505 nm and at 535 nm. Adding 1 μM valinomycin induced ~10% decrease in absorbance maxima of the dye, together with a blue shift of 6 nm from 505 nm to 499 nm.

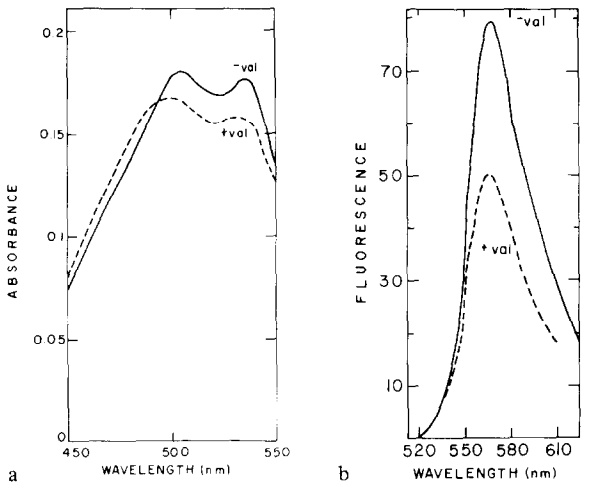

Fig.1(a) Effect of valinomycin on the absorption spectrum of MC-540. Fig.1(b) Effect of valinomycin on the emission spectrum of MC-540. Buffer: 153 mM KCl; 17 mM Tris–HCl (pH 7.4). Excitation wavelength: 505 nm. (——) 2.9 μM, MC-540 alone. (– – –) 2.9 μM MC-540 + 1 μM valinomycin. Spectra were recorded 5 min after adding the valinomycin.

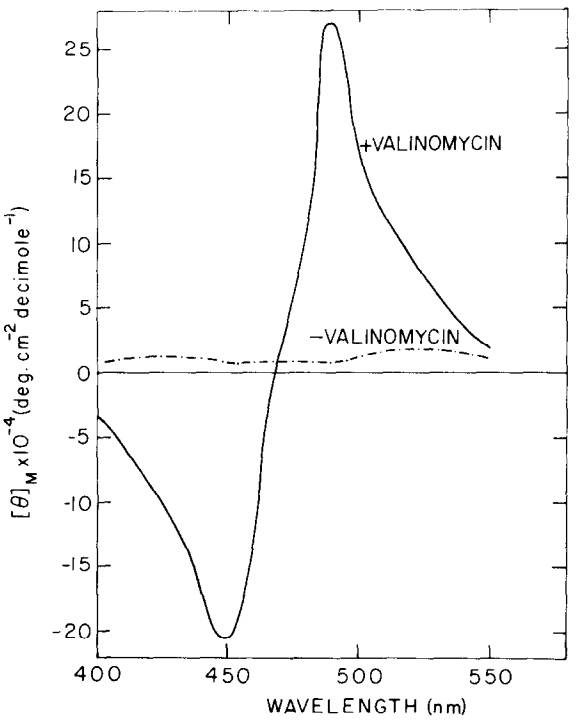

Fig.2. Optical activity of MC-540 induced by valinomycin in the presence of high K^+. (–.–.) 2.9 μM MC-540 alone. (——) 2.9 μM MC-540 + 1 μM valinomycin. Spectrum was recorded 5 min after adding the ionophore. Buffer: 153 mM KCl; 17 mM Tris–HCl (pH 7.4). Valinomycin alone and MC-540 + valinomycin in 153 mM NaCl showed no significant optical activity between 400–550 nm.

Valinomycin, in the absence of MC-540, did not show any absorbance or fluorescence between 420–550 nm. The MC-540 emission spectrum revealed an even more marked effect of valinomycin (fig.1(b)). The fluorescence peak at 575 nm was shifted by adding valinomycin to 570 nm and the peak intensity was reduced by 40%. The effects of valinomycin on MC-540 absorbance or fluorescence, in low K^+ (5 mM) medium, were much smaller (data not shown). CD measurements show that the MC-540 (2.9 μM) alone, in high K^+ medium, has very low optical activity in the visible range (fig.2). However, when 1 μM valinomycin was added, the formation of two large and almost equal bands was observed. The two bands had opposite signs: a positive maximum centered at 492 nm and a negative one at 450 nm. The appearance of the CD signals was observed immediately after adding the valinomycin and reached maximum magnitude in 3 min. The absorbance and emission changes followed similar kinetics. Addition of valinomycin in the presence of 153 mM Na^+ had little effect on the MC-540 CD spectrum. Valinomycin alone, in high K^+ solution, showed no significant optical activity in the visible range.

Similar experiments were performed by using the carbocyanine dye $DiSC_3(5)$. In the presence of 153 mM K^+, valinomycin had no effect on the visible absorbance spectrum of $DiSC_3(5)$. There was a minor absorption peak at 590 nm and a major absorption peak at 650 nm (data not shown). The ionophore was without any effect on the fluorescence emission of this dye (not shown). No optical activity was observed for $DiSC_3(5)$ either in the absence or presence of valinomycin.

These differences between MC-540 and $DiSC_3(5)$ are well reflected on studying the fluorescence response to valinomycin in human erythrocyte suspensions. An action of valinomycin on the human erythrocyte-$DiSC_3(5)$ interaction is demonstrated in two extreme cases (fig.3(a,b)). On adding valinomycin in 153 mM K^+ medium (fig.3a), a 10% increase in the maximum emission (670 nm) of $DiSC_3(5)$ was observed. This increase was referred to in [3] as depolarization of the cell causing release of the dye from the

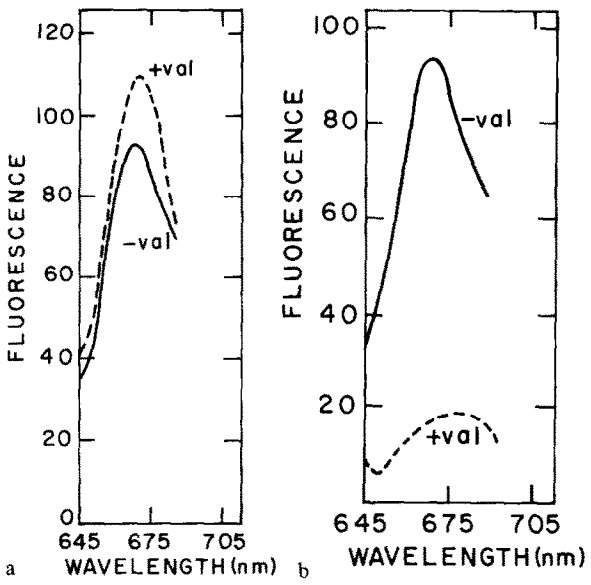

Fig.3a. Depolarization of human erythrocyte as reflected in $DiSC_3(5)$ emission fluorescence. Buffer contained: 153 mM KCl, 17 mM Tris–HCl (pH 7.4). Erythrocyte conc.: 0.33%. Fig.3b. Hyperpolarization of human erythrocyte as reflected in $DiSC_3(5)$ emission spectrum. Buffer contained: 153 mM NaCl, 17 mM Tris–HCl (pH 7.4). (——) Cells + 0.61 μM $DiSC_3(5)$. (– – –) Cells + 0.61 μM $DiSC_3(5)$ + 1 μM valinomycin. Excitation wavelength: 622 nm.

cell to the membrane. In fig.3(b), in the presence of 153 mM Na^+ and no K^+ the valinomycin induced an 80% drop in the fluorescence maximum of $DiSC_3(5)$. This effect occurred within a few seconds, and is explained by hyperpolarization which is reflected in an increase of dye uptake by the cell [3]. Others have demonstrated a linear correlation between $DiSC_3(5)$ fluorescence and the logarithm of the $[K^+]$ [4].

In contrast to the pronounced sensitivity of $DiSC_3(5)$ to the K^+-equilibrium potential of the red cells, the spectral changes observed with MC-540 were independent of the presence of erythrocytes.

This report brings spectroscopic evidence for interaction between MC-540 and K^+-valinomycin in aqueous solution. This 'complex' shows altered absorption and emission spectra (fig.1(a,b)). It shows large CD bands in the visible region (fig.2). The appearance of optical activity may indicate that K^+-valinomycin, upon interacting with MC-540, affects the symmetry of the dye, either by distorting the planarity of the monomer dye molecule, or by inducing aggregation of MC-540. The aggregates of the dye have been shown [11] to have different spectral properties than the monomer. The spectroscopic studies and optical activity measurements clearly show that free $DiSC_3(5)$ does not interact with valinomycin even in the presence of high $[K^+]$. The difference between the two dyes may be due to charge differences between the negatively-charged MC-540 and the positively-charged $DiSC_3(5)$. In fact, ANS and other organic anionic compounds have also been reported to interact with valinomycin-cations in water [8,12]. The similar effects of valinomycin on MC-540 fluorescence, in the absence (fig.1(b)) and in the presence of cells, indicate that in the latter case the fluorescence change emerges mainly from interaction between the free dye and ionophore and is not associated with membrane potential changes. This interaction may interfere with the normal action of the ionophore on the cells, namely on the K^+-diffusion potential. Similar difficulties using ANS in combination with valinomycin have been reported [12]. We show here that free $DiSC_3(5)$ does not interact with the ionophore. Thus, the valinomycin-induced $DiSC_3(5)$ fluorescence changes, observed with erythrocytes (fig.3(a,b)) may mostly be related to membrane potential events.

In summary, we draw attention here to possible pitfalls in using certain membrane-potential probes together with ionophores like valinomycin. It is worth noting that MC-540 is considered advantageous in some systems [13] over carbocyanine because of its ability to respond rapidly with larger signal to noise ratios. Therefore, we feel it is important to apply further efforts to try to calibrate MC-540 by other means, possibly by manipulation of the ionic composition of the system without adding ionophores.

Acknowledgements

E. L. acknowledges financial support from a fellowship of the Anna Fuller Fund. The authors are grateful to Professor Kurt Rosenheck for helpful discussions.

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